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Clearance of Rh-Positive Red Cells by Low Concentrations of Rh Antibody

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Summary. Ten previously untransfused Rh(D)-negative subjects were given an intravenous injection of approximately 0·3 ml of Rh(D)-positive red cells and at about the same time, or 24–48 hours previously, were given an intramuscular injection of 1–1000 μg of anti-D.

Following the injection of the red cells there was a variable period before the onset of red cell destruction; when the antibody was injected at the same time as the red cells the delay was due partly to the time taken for the antibody to reach the circulation; when the antibody was injected at least 24 hours before the red cells there was still some delay due to the time required for the antibody to be taken up by the red cells; the delay before the onset of a maximum rate of red cell destruction varied from about 0·2 hours following the injection of 1000 μg of antibody to approximately 100 hours following the injection of 1 μg.

The maximum rate of red cell destruction was calculated to be approximately proportional to the square root of the amount of antibody on the cells. After the injection of the smallest amount of antibody (1 μg) the amount on the red cells was calculated to be about 0·03 μg/ml corresponding to about ten molecules of antibody per cell. This dose produced clearance with a $T_{\frac{1}{2}}$ of the order of 100 hours.

The significance of these observations in relation to protection against Rh-immunization is briefly discussed.

INTRODUCTION

Suppression of the antibody response by injecting antibody and antigen simultaneously was first described by Smith in 1909 and has recently been studied by many investigators (e.g. Finkelstein and Uhr, 1964). The possibility of suppressing the antigenic response to Rh-positive red cells by injecting Rh antibody simultaneously was first demonstrated by Stern, Goodman and Berger (1961) who found that Rh-sensitized red cells were far less antigenic than unsensitized Rh-positive red cells. Subsequently, it was shown by others that if the injection of Rh-positive red cells was followed by an injection of a potent Rh antibody, active immunization was prevented (Clarke, Donohue, McConnell, Woodrow, Finn, Krevans, Kulke, Lehane and Sheppard, 1963; Freda, Gorman and Pollack, 1964).

The observations have recently found an important practical application for it has been shown that it may be possible to prevent Rh-immunization which would otherwise occur as a result of pregnancy. Thus it has been found that the injection of Rh antibody, within 36 hours of delivery, to Rh-negative women who have 0·2 ml or more of Rh-positive foetal

red cells in their circulation, almost abolishes the risk that they will form detectable amounts of antibody within the following 6 months (Clarke, Finn, Lehane, McConnell, Sheppard and Woodrow, 1966). Similar observations have been reported by Freda *et al.* (1966).

If Rh antibody is to be given as a routine prophylactic measure to recently delivered Rh-negative women, it is evidently desirable to discover the least amount of antibody which is effective. There seems to be some danger in giving relatively small amounts of γM antibody, since this may actually result in enhanced antibody formation (Clarke *et al.*, 1963). On the other hand, amounts of potent γG anti-Rh as small as 0·0001 ml, given with a small amount of Rh-positive red cells, do not seem to have any enhancing effect (Freda *et al.*, 1966, supplemented by personal communication). The giving of unnecessarily large amounts of Rh antibody is contra-indicated, mainly because of limited supplies but also because it seems desirable not to inject more foreign protein than is essential.

The present work was designed to discover more precisely the amounts of Rh antibody required to bring about the clearance of small numbers of Rh-positive red cells at particular rates, in the hope that this knowledge would ultimately prove useful in predicting the dose necessary to prevent sensitization.

METHODS

Subjects

Rh(D) negative subjects who had originally offered their services as blood donors, but had been rejected because of a previous history of jaundice, were invited to take part after the risks of the procedure had been fully explained to them; one woman who had passed the menopause, and nine men took part.

Injection of Rh-positive red cells

The same group O, R_1r, K-donor was used throughout; she was a healthy young woman who had served as a donor on many previous occasions without, so far as could be ascertained, transmitting serum hepatitis. Samples of red cells were labelled with ^{51}Cr after being washed in citrate–phosphate pH 6·9 (Mollison, 1961, p. 162). The total amount of red cells injected into each subject was determined by estimating the haemoglobin concentration of the injection suspension and varied from 0·29 to 0·42 ml with an average of 0·34 ml.

γ-Globulin containing anti-Rh (D)

A single batch was used, prepared by Dr L. Vallet in 1964 by ethanol-fractionation from a pool of 5 l of serum from a female donor. The donor had originally been immunized by pregnancy and had been given booster injections of Rh-positive red cells before being bled. During the period of venesection the titre of Rh antibody, determined by the indirect antiglobulin test (as described by Mollison, 1961, p. 411) varied from 2000 to 10,000; the titre of the γ-globulin preparation was approximately 30,000. The amounts of γ-globulin injected into volunteers ranged from 0·004 to 4·0 ml and were given intramuscularly. Amounts in the range 0·2–4 ml were given as undiluted γ-globulin; for the smaller amounts dilutions of the γ-globulin were prepared in saline.

Anti-D content of γ-globulin

The concentration, equilibrium constant and index of heterogeneity (*a*) were deter-

mined by the method of Hughes-Jones (1967), using [^{125}I]anti-γ globulin, which was calibrated against purified [^{131}I]anti-D from the same donor.

Plan of experiments (Table 1)

In seven cases the [^{51}Cr]Rh-positive red cells were injected first; after taking a blood sample (in duplicate) at 10 minutes the γ-globulin was injected. In three cases γ-globulin was injected either 24 or 48 hours before the injection of the red cells.

TABLE 1

DETAILS CONCERNING THE RELATIONSHIP BETWEEN THE AMOUNT OF ANTI-D INJECTED AND THE MAXIMUM RATE OF RED CELL DESTRUCTION

Subject	Injection of anti-D		Maximum rate of red cell destruction	
	Amount (μg)	Time before injection of red cells	Delay before onset (hours)	$T_{\frac{1}{2}}$
Pr	1000	0	2–4	—
		24 hours	0·2	1·5
Ty	250	24 hours	1·2	1·5
Br	250	0	5	2·5
Ed	50	0	13	5·0
Ke	50	0	9	5·5
Ro	50	48 hours	3·6	3·0
Os	12·5	0	17	8·0
	(4)*	42 days	45	23
Gr	5	0	30	9·5
	(1·5)*	43 days	70	144
Su	5	0	30	13·5
	(1·9)*	36 days	109	52
Ha	1	24 hours	105	53

* Amount of anti-D calculated to be left in body 36–43 days after injection of γ-globulin.

In four of the ten volunteers a second injection of [^{51}Cr]Rh-positive cells was given at a time when the red cells from the first injection had been cleared from the circulation.

Blood samples were taken from the recipient at various intervals in order to determine: (1) the time of onset of red cell destruction, and (2) the maximum rate of clearance achieved. For the convenience of the volunteers the number of samples taken was far less than optimal but it was possible in all cases to make reasonably satisfactory estimates.

Blood samples taken from each recipient were counted as a batch in a well-type scintillation counter. In all cases the results were expressed as ^{51}Cr/g of haemoglobin after determining the haemoglobin concentration of the samples, and taking into account the presence of small amounts of Cr in the plasma. The results were corrected for Cr elution (see Mollison, 1961, p. 162).

Indirect antiglobulin tests

Samples taken from the recipient approximately 24 hours after the injection of γ-globulin were examined by the indirect antiglobulin test against Rh-positive and Rh-negative red cells. In the standard method used in this laboratory (Mollison, 1961, p. 411)

four volumes of serum are incubated with one volume of a 20 per cent suspension of red cells so that the ratio of serum to cells is 20:1. In the present work in order to increase the sensitivity of the test the ratio of serum to cells was increased to 1000:1 and the results were compared.

RESULTS

OBSERVATIONS ON Rh ANTIBODY

Characteristics of anti-D

The estimate of the anti-D content of the γ-globulin solution was 250 μg/ml. The average equilibrium constant (K) was $1{\cdot}6 \times 10^8$ l/mole and the heterogeneity index, $a = 0{\cdot}7$ (see 'Discussion').

Amount of antibody on the D-positive red cells in vivo *at equilibrium*

This could not be directly estimated as the number of cells injected was too small. An estimate was obtained, however, from a calculation using the average equilibrium constant and the index of heterogeneity according to the equation given by Karush(1962), assuming that: (1) R_1r cells which were injected had 12,000 D sites/red cell (Rochna and Hughes-Jones, 1965), and (2) 50 per cent of the injected anti-D was present in the plasma at the time of destruction of the red cells. These calculated values for the amount of antibody on the red cells are those used in Fig. 4.

Anti-D content of recipients' plasma

The anti-D content of plasma was estimated in two recipients who had received 1000 and 250 μg 24 hours previously and was found to be 0·2 and 0·06 μg/l respectively; these amounts corresponded to the presence in the plasma of approximately 60 and 72 per cent of the injected dose.

Indirect antiglobulin tests on recipients' plasma

Using a serum–red cell ratio of 20:1, anti-D could just be detected in the plasma of the two subjects who had received 250 μg of antibody but could not be detected in the plasma of the subjects who had received smaller doses. Using a serum–red cell ratio of 1000:1 Rh antibody was readily detected in two out of the three subjects who had received 50 μg but could not be detected in any of the subjects who had received smaller doses.

RATE OF DESTRUCTION OF D-POSITIVE RED CELLS

The relationship between the total amount of anti-D injected and the $T_{\frac{1}{2}}$ for the maximum rate of destruction is given in Table 1 and Figs. 1, 2 and 3.

There was substantial agreement between cases in which similar doses of anti-D were given. The relationship between the rate of destruction ($T_{\frac{1}{2}}$) and the amount of antibody calculated to be present on the cells is given in Fig. 4.

Re-injection of cells 6 *weeks after anti-D injections*

Three recipients were given a second injection of 0·3–0·4 ml of D-positive red cells 6 weeks after the initial injection of 5, 5 and 12·5 μg of anti-D. In all three cases, the $T_{\frac{1}{2}}$ for the maximum rate of removal of red cells was considerably longer than that seen with

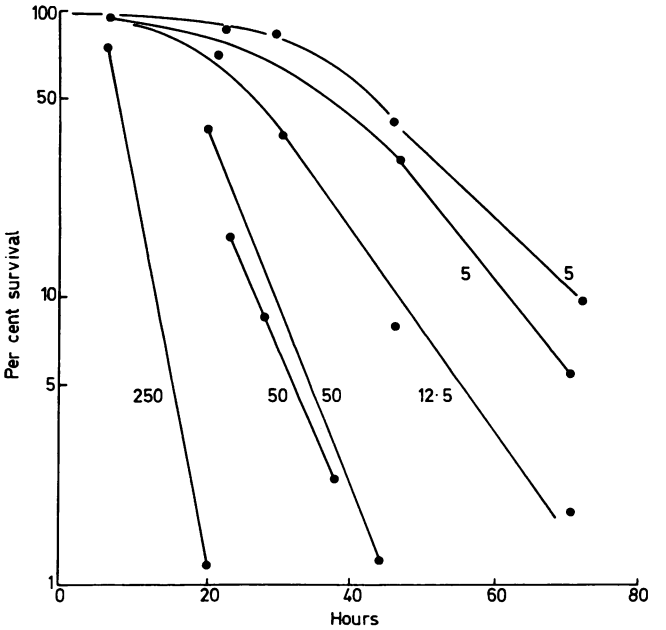

FIG. 1. Survival of 0·3 ml of Rh-positive red cells in six previously untransfused Rh-negative males; 10 minutes after the injection of the red cells each subject received an injection of γ-globulin containing anti-D; the figures against the curves indicate the various amounts of antibody injected in μg.

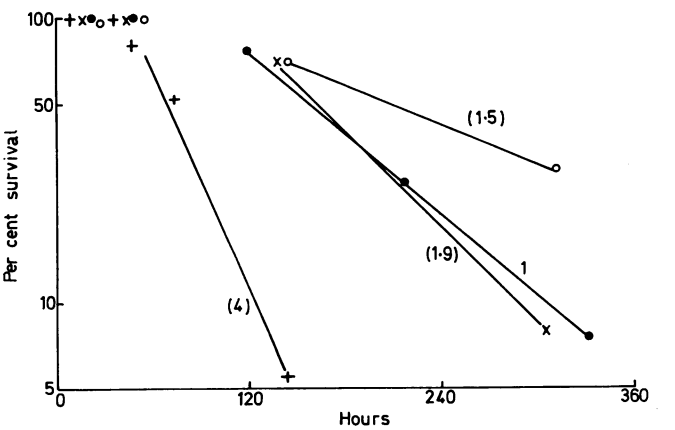

FIG. 2. Survival of 0·3 ml of Rh-positive red cells in four Rh-negative subjects; three of the subjects had received a similar injection of red cells together with an injection of anti-D containing γ-globulin 36–43 days previously. The approximate amounts of anti-D (in μg) calculated to be remaining in the circulation at the time of the second injection are shown in parentheses. The fourth subject had not been injected with red cells previously but had received an injection of 1 μg of anti-D 24 hours before being injected with red cells.

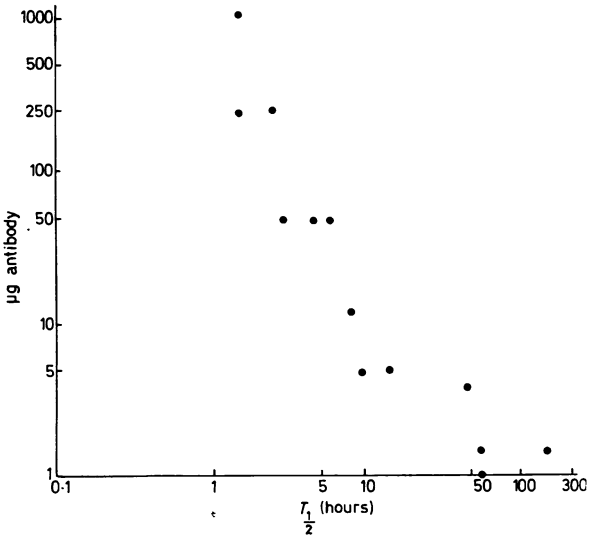

FIG. 3. Rates of destruction of Rh-positive red cells shown as a $T_{\frac{1}{2}}$ (hours) plotted against the total amount of anti-D injected (as µg antibody).

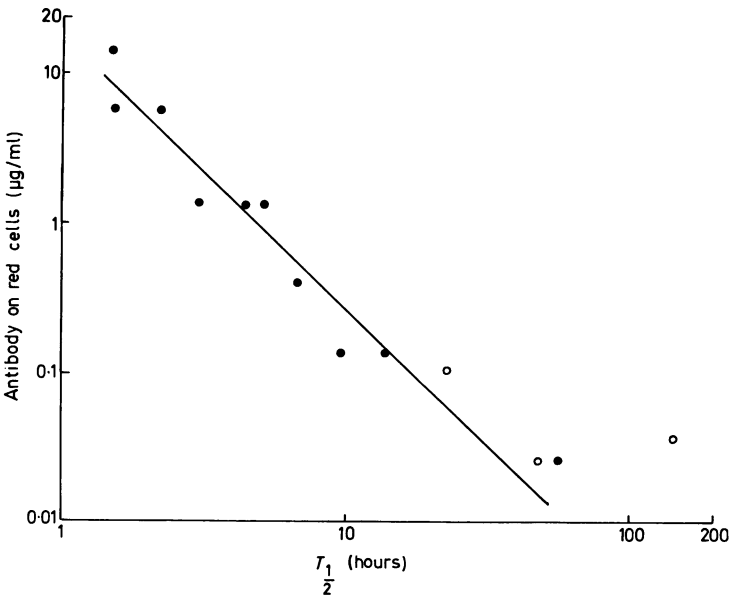

FIG. 4. Observed rate of destruction of D-positive red cells as $T_{\frac{1}{2}}$ plotted against the calculated amount of anti-D on red cells, derived from the amount of anti-D injected and from formulae based on the law of mass action. ●, Cells injected within 24 hours of anti-D administration; ○, cells injected 36–43 days after anti-D.

the initial injection of cells. If the assumption was made that the anti-D had survived in the plasma with a $T_{\frac{1}{2}}$ of 21 days, then the concentration present at the time of the second injection of cells could be calculated and from this an estimate made of the amount of anti-D combined with the red cells. It can be seen (Fig. 2) that the rate of destruction agrees reasonably well with that expected from the amount of anti-D remaining in the circulation. There is then no evidence for the production of anti-D by these three recipients 6 weeks after the injection of 0·3–0·4 ml of D-positive red cells.

Delay before the onset of red cell destruction

In all cases following the injection of Rh-positive red cells there was an initial period in which there was no measurable destruction. In the cases in which the γ-globulin had been injected at least 24 hours before the injection of the Rh-positive cells the delay in the onset of red cell destruction was evidently due solely to the relatively slow uptake of antibody onto the red cells. By backward extrapolation of the steepest part of the survival curve it was possible to estimate the delay before the onset of the maximum rate of red cell destruction and this was found to vary between about 12 minutes in the case in which 1000 μg of antibody had been injected 24 hours previously to about 4 days in a case in which only 1 μg had been injected (see Figs. 2 and 5). Similar long delays before the onset of red cell destruction were observed by Schneider and Preisler (1966), after injecting 1 ml of D-positive cord blood intravenously and low-titred anti-D globulin intramuscularly, into Rh-negative subjects.

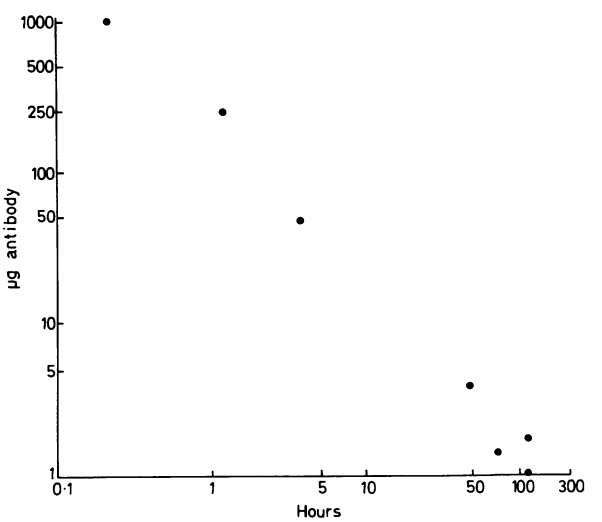

FIG. 5. Observed delay between the injection of Rh-positive red cells and the onset of an approximately maximal rate of red cell destruction, plotted against the amount of anti-D injected. In all cases the anti-D was injected 24 hours or more before the injection of the red cells.

In the cases in which γ-globulin was injected 15 minutes after the injection of the Rh-positive cells, there was an additional delay due to the time taken for the uptake of γ-globulin into the circulation. By comparing cases in which the same amount of γ-globulin was injected into different subjects either 24 hours previously or 15 minutes after the red cells, it was estimated that the time between the injection of γ-globulin and the build-up

in the blood stream of a substantial concentration of γ-globulin was approximately 6 hours (Fig. 6).

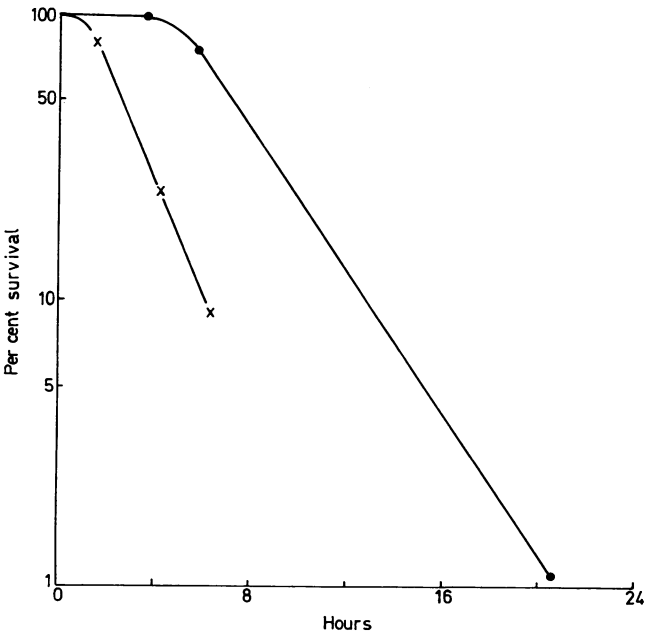

FIG. 6. Destruction of 0·3 ml of Rh-positive red cells in two subjects injected with 250 μg anti-D. ●, Anti-D injected 10 minutes after red cells; ×, anti-D injected 24 hours before red cells.

DISCUSSION

The data presented in Fig. 3 show that the rate of destruction is not directly proportional to the amount of antibody injected into the recipients. Thus, decreasing the amount of anti-D injected by a factor of 200 (from 1000 to 5 μg) only prolongs the $T_{\frac{1}{2}}$ by a factor of approximately 10. This is partly due to the fact that the percentage of antibody adsorbed onto the red cells increases as the amount of injected antibody decreases. The percentage of antibody adsorbed depends on two factors: (1) the average value and extent of heterogeneity of the equilibrium constant, and (2) the number of red cells present in the circulation. Using the estimated values of K and a, it was calculated that the maximum uptake of anti-D by 0·35 ml of cells was approximately 1 per cent of the total antibody injected.

When the rate of destruction is correlated with the calculated amount of antibody on the cells (Fig. 4), it is found that a 100-fold decrease in the amount of antibody on the cells brings about approximately a ten-fold prolongation in $T_{\frac{1}{2}}$; that is, the rate of destruction is approximately proportional to the square root of the amount of antibody on the cells. It should be noted that this assumes that, at the time when the maximal rate of red cell destruction was occurring, the concentration of antibody in the circulation had reached a maximum. The observations of Freda *et al.* (1964) suggest that when antibody in the form of γ-globulin is injected intramuscularly, the concentration in the serum reaches a maximum within about 8 hours. On the other hand, observations with [^{131}I]-globulin indicate that following intramuscular injection, a maximum concentration,

corresponding to the presence in the circulation of about 40 per cent of the injected γ-globulin, is not reached for 48 hours, although 50 per cent of the final concentration is reached within 24 hours (Du Pan, Wenger, Koechili, Scheideggar and Roux, 1959). If it is true that uptake of antibody is not complete within 24 hours following intramuscular injection, then the calculations shown in Fig. 4 may overestimate, by a factor of approximately 2, the amount of antibody on the cells when the larger doses were given; that is, the red cell destruction occurred before the maximum concentration of antibody in the circulation had been reached. Nevertheless, the main conclusion that small numbers of antibody molecules have a relatively greater effect than larger numbers in bringing about red cell destruction appears to be valid.

The remarkable biological potency of this particular example of anti-D is demonstrated by the observation that the injection of only 1 μg of antibody (plasma concentration approximately 1×10^{-12} M), brings about the destruction of red cells. The concentration of antibody adsorbed onto the red cells in this instance was calculated to be 0·033 μg/ml red cells (i.e. 0·01 μg on 0·3 ml cells), which corresponds to approximately ten molecules of anti-D per red cell. The ability of this example of anti-D to combine with red cells in sufficient amounts to bring about their destruction is due in part to the heterogeneity of the equilibrium constant. The precise distribution of the equilibrium constants is not known, but the index of distribution used here is that initially proposed by Sips (1948) and is almost identical to a Gaussian distribution. It has not yet been definitely shown that the distribution of K can be described by a Gauss error function, but the antigen–antibody reactions that have been studied give data which are consistent with this view (Nisonoff and Pressman, 1958; Karush, 1962; Eisen and Siskind, 1964; Hughes-Jones, 1967). The effect of heterogeneity is to increase the percentage of antibody bound to cells, especially at low concentrations. This can be illustrated by the fact that if the particular anti-D which we investigated here had been homogeneous then it can be calculated that on an average only one molecule of anti-D would have combined with each red cell after the injection of 1 μg of antibody. If the Sips index of heterogeneity (a) does in fact represent the true distribution, then it can be shown (Fujio and Karush, 1966) that an antibody with an index of $a = 0{\cdot}7$ and an average value of $K = 1{\cdot}6 \times 10^8$ l/mole will have approximately 2 per cent of the molecules with a value of K greater than 10^{10} l/mole and it is predominately these molecules with the relatively high binding constants that combine with the red cells at low concentrations and raise the number of bound molecules from one to approximately ten.

Examples of anti-D have different values of K and different indices of heterogeneity (Hughes-Jones, 1967); hence the biological effectiveness of antibodies will differ when compared on a molar basis. Furthermore, it is likely that the ability of an antibody to bring about phagocytosis is dependent on some specific part of the structure of the γ-globulin and that this may also show heterogeneity. A further experiment was carried out which indicates that these considerations are probably correct. A recipient was injected with 150 μg of another anti-D (Wa) which had a value of K of only one-fifth ($K = 3{\cdot}3 \times 10^7$ l/mole) of the anti-D used in the main experiment. This antibody (Wa), brought about the destruction of red cells with a $T_{\frac{1}{2}}$ of 15 hours, a rate considerably slower than that expected with a comparable amount of anti-D (Avg) (Table 1). The discrepancy could be partly accounted for by the fact that less anti-D (Wa) was taken up by the cells owing to the lower value of the equilibrium constant, but even when this was taken into account, the rate of destruction with anti-D (Wa) was approximately three

times slower than with anti-D (Avg) when similar amounts of antibody were present on the cells. Although biological variation between recipients in the activity of their respective reticulo-endothelial systems cannot be ruled out, it is more probable that there is a variation in the ability of these two antibodies to bring about phagocytosis.

Some previously published observations strongly support this belief. In a case described by Mollison and Cutbush (1955), following the injection of 1 ml of c-positive red cells into the circulation of a subject whose serum contained anti-c with a titre of 8, the cells were removed from the circulation with a half-time of 7 days; it may safely be assumed that the amount of antibody on the circulating red cells was of the order of a few μg/ml; a comparable amount of the anti-D(Avg) used in the present series of experiments would produce clearance with a $T_{\frac{1}{2}}$ of a few hours. An even more striking example of an ineffective antibody was described by Sausais, Krevans and Townes (1966); the antibody had the specificity anti-Xg^a and was detectable by the indirect antiglobulin test, but it failed completely to diminish the survival of transfused Xg(a+) red cells.

The finding that after the injection of 1 μg of antibody there was a lag period of approximately 100 hours before the onset of destruction of cells can be shown to be of the order of time expected from calculations based on *in vitro* observations. The rate constant for association for anti-D (Avg) with R_1R_1 cells has been determined to be approximately 1×10^5 l/mole/sec, at 37° (Hughes-Jones, Gardner and Telford, 1963). Using this value it can be calculated that the reaction between antibody and antigen should be approximately 95 per cent complete after 50 hours, a value less by a factor of 2 than the observed lag period, but within the experimental error of the methods used in the estimates.

The findings presented in Fig. 2 suggest that the injected anti-D was catabolized faster than normal γG-globulin. Thus the rates of clearance of Rh-positive red cells injected several weeks after the injection of antibody were slightly less than expected if the anti-D had been catabolized with a $T_{\frac{1}{2}}$ of 21 days. In two further cases, not referred to elsewhere in this paper, in which an injection of Rh-positive cells was given 20–22 weeks after the initial injection of antibody, the rate of clearance was that expected if the antibody had been broken down with a $T_{\frac{1}{2}}$ of 16 days. Since this $T_{\frac{1}{2}}$ is appreciably less than that expected from the normal $T_{\frac{1}{2}}$ of catabolism of γG-globulin it must be supposed that fractionation of the anti-D serum, or prolonged storage at $-20°$, caused some damage to the antibody molecules.

The experiments reported here have emphasized that antibody concentrations far below those that can be detected *in vitro* are capable of bringing about red cell destruction. The limit of sensitivity of the indirect antiglobulin (anti-γG) test using a serum: red cell ratio of 20:1 is usually in the range 0·05–0·5 μg antibody/ml (unpublished observations); even when the serum: red cell ratio is increased to 1000:1, the lowest detectable concentration is probably then about 0·01 μg/ml. By contrast, the lowest concentration of antibody bringing about red cell destruction in the present experiments was approximately 0·0002 μg/ml, about fifty times less than the lowest concentration detectable by the indirect antiglobulin test *in vitro*.

At the same time it must be remembered that these conclusions refer to the transfusion of very small amounts of red cells and that the discrepancy between the sensitivity of tests *in vitro* and red cell survival *in vivo* is much less when therapeutic amounts of blood are transfused.

The mechanism whereby passively administered antibody suppresses active immunization is unknown. Some workers have assumed that rapid clearance of antigen from the circulation is the key factor and that, for example, this is the mechanism whereby ABO

incompatibility protects against Rh-immunization (Clarke *et al.*, 1963; Freda *et al.*, 1966). It is possible that protection by ABO-incompatibility is due to the fact that either intact or lysed red cells are taken up mainly by the macrophages in the liver and that the liver is an unfavourable site for antibody formation. Protection by Rh antibody seems unlikely to depend on the fact that the antibody brings about rapid removal of red cells from the circulation since Rh antibodies produce sequestration of red cells predominantly in the spleen which is known to be a highly favourable site for antibody production.

It is unlikely that protection is due to masking of antigen sites, since protection has been achieved in conditions in which only a small percentage of the sites would have been occupied. For example, Clarke *et al.* (1963) reported that protection was achieved by giving 50 ml of Rh antibody with a titre of approximately 1000 together with an injection of 5 ml of Rh positive red cells; the red cells were cleared from the circulation within 24 hours. It can be shown that the removal of 99 per cent of red cells within 24 hours, corresponding to a rate of clearance of 0·32 per cent per minute, requires the occupancy of only about 10 per cent of the antigen sites. It seems more likely that passively administered antibody prevents immunization by diverting antigen from antibody-forming cells; this diversion may occur after the red cells have been broken down in macrophages. If so, the rate at which the red cells are destroyed may be less important than the ratio of passively administered antibody molecules to antigen molecules throughout the period of persistence of antigen. Nevertheless, it seems likely that the rate of red cell clearance and the degree of suppression of the primary response will prove to be closely related, and that knowledge of the rate of red cell clearance produced by a particular dose of antibody will make it possible to predict, within limits, the amount required to suppress primary immunization.

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